Machine perfusion of human donor livers with a focus on the biliary tree

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CHAPTER

Ex Situ Normothermic Machine Perfusion of Donor Livers

(Video Article)

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ABSTRACT

In contrast to conventional static cold preservation (0-4 °C), *ex situ* machine perfusion may provide better preservation of donor livers. Continuous perfusion of organs provides the opportunity to improve organ quality and allows *ex situ* viability assessment of donor livers prior to transplantation. This video article provides a step by step protocol for *ex situ* normothermic machine perfusion (37 °C) of human donor livers using a device that provides a pressure and temperature controlled pulsatile perfusion of the hepatic artery and continuous perfusion of the portal vein. The perfusion fluid is oxygenated by two hollow fiber membrane oxygenators and the temperature can be regulated between 10 °C and 37 °C. During perfusion, the metabolic activity of the liver as well as the degree of injury can be assessed by biochemical analysis of samples taken from the perfusion fluid. Machine perfusion is a very promising tool to increase the number of livers that are suitable for transplantation.

INTRODUCTION

The current method of organ preservation in liver transplantation is flush out with and subsequent storage of donor livers in cold (0-4 °C) preservation fluid (such as University of Wisconsin solution or Histidine-Tryptophan-Ketoglutarate solution). This method is referred to as static cold storage (SCS). Although the metabolic rate of livers at 0-4 °C is very low, there is still demand for 0.27 µmol oxygen/min/g liver tissue, which cannot be provided during SCS.[1] The conventional method of SCS, therefore, results in some degree of (additional) injury of donor livers. While this amount of preservation injury is not a problem in donor livers of good quality, it can become a critical and limiting factor in suboptimal livers that have already suffered some degree of injury in the donor. For this reason, livers with suboptimal quality or so-called extended criteria donor (ECD) livers are frequently rejected for transplantation as the risk of early graft failure is considered to be too high. High rates of delayed graft function, primary non-function, and non-anastomotic biliary strictures (NAS) have been described in recipients of livers from donation after circulatory death (DCD), older donors or recipients of steatotic grafts.[2] NAS are a major cause of morbidity and mortality after liver transplantation. NAS may occur in both extra- and intrahepatic donor bile ducts and can be accompanied by intraductal biliary sludge and cast formation.[3,4] Although the etiology of NAS is thought to be multifactorial, ischemia/reperfusion injury of the bile ducts during graft preservation and transplantation has been identified as a major underlying mechanism.[2,5] Transplantation of a DCD graft has been identified as one of the strongest risk factors for the development of NAS. The combination of a period of warm ischemia in a DCD donor, cold ischemia during organ preservation, and subsequent reperfusion injury in the recipient is thought to be responsible for irreversible injury of the bile ducts, which, in combination with a poor regenerative capacity of the bile ducts, results in fibrotic scarring and narrowing of the bile ducts after liver transplantation.[2,5] NAS have been reported in up to 30% of patients receiving a DCD liver.[6-8] It has become clear that the current method of SCS of liver grafts for transplantation is insufficient for pre-injured ECD livers such as those from DCD donors. Alternative methods are needed to increase and optimize the use of ECD livers for transplantation.

Machine perfusion (MP) is a method of organ preservation that may provide better preservation of donor organs, compared to SCS. MP could be especially relevant for the preservation of ECD grafts. An important advantage of MP is the possibility to provide oxygen to the graft during the preservation period. MP can be performed at various temperatures, which have been classified as hypothermic (0-10 °C), subnormothermic (10-36 °C) and normothermic (36-37 °C) MP (NMP). Depending on the temperature used for MP, the type of perfusion fluid has to be adjusted and with increasing temperature more oxygen should be supplied. The first clinical application of MP in human liver transplantation was based on hypothermic perfusion without active oxygenation of the perfusion fluid.[9,10] In animal models, hypothermic oxygenated MP (0-10 °C) has been shown to have protective effects against

ischemia/reperfusion injury of liver grafts[11] and to provide better preservation of the peribiliary vascular plexus of the bile ducts.[12] Subnormothermic oxygenated MP at 20 °C or 30 °C has also been studied in animal models and was shown to provide earlier recovery of graft function of DCD livers, compared to SCS.[13,14] The feasibility of subnormothermic oxygenated MP of human livers was recently reported in a series of seven discarded human donor livers.[15] NMP (37 °C) allows for the assessment of graft viability and functionality prior to transplantation.[16,17] Additionally, MP allows for gradual rewarming of the liver graft before transplantation, which has been demonstrated to facilitate recovery and resuscitation of the graft.[18]

The perfusion device used in the current protocol for hepatic machine perfusion enables dual perfusion (via the portal vein and the hepatic artery) using two centrifugal pumps, that provide a continuous portal flow and a pulsatile arterial flow. The system is pressure-controlled, allowing auto-regulation of the flow through the liver, depending on the intrahepatic resistance. Two hollow fiber membrane oxygenators allow for the oxygenation of the liver graft, as well as for the removal of CO_2. The temperature can be set based on the intended type of MP (minimum temperature of 10 °C). Flow, pressure and temperature are displayed on the device in real-time allowing a continuous control of the perfusion process. A new sterile disposable set of tubing, reservoir and oxygenators is available for the perfusion of each graft (**Figure 1**).

The aim of this video article is to provide a step by step protocol for *ex situ* normothermic machine perfusion of human donor livers using this newly developed liver perfusion machine.

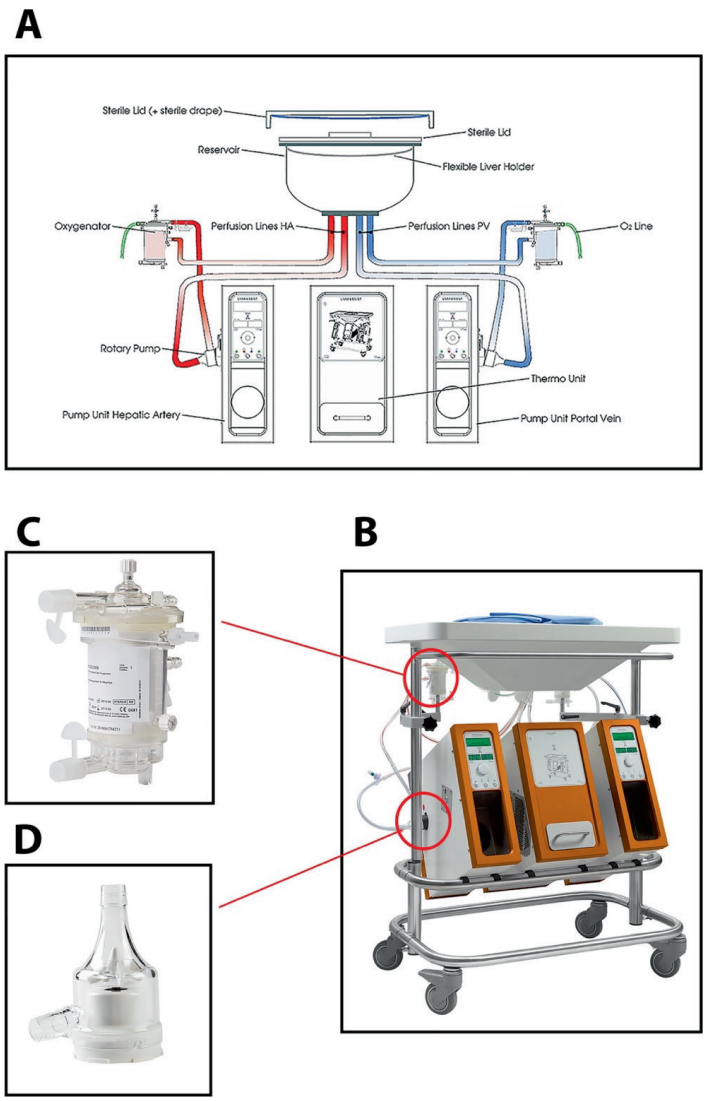

Figure 1: (**A**) A schematic drawing, (**B**) a photo of the perfusion machine, (**C**) a closer view of the oxygenator, and (**D**) centrifugal pump used for normothermic perfusion of human donor livers.

PROTOCOL

This protocol has been approved by the Medical Ethical Committee (Medisch Ethische Toetsingscommissie) of the University Medical Center Groningen, the Netherlands.

1. Preparation of the Perfusion Fluid

Note: The total volume of the perfusion fluid prepared for normothermic machine perfusion according to this protocol is 2,233 ml and the targeted osmolarity of the perfusion fluid is 302 mOsmol/L.

a. From the components of the perfusion fluid described in **Table 1**, keep the human packed red blood cells, fresh frozen plasma and human albumin separated. Mix the rest of the components in a sterile manner and store the solution in a sterile bag for transportation to the operating room (OR). Do this in a sterile environment (ideally a Good Manufacturing Practice facility) or in a laminar flow cabinet in a culture room.
b. Transfer human packed red blood cells (840 ml), fresh frozen plasma (930 ml), human albumin 200 g/L (100 ml) and the solution prepared in step 1.1 to the OR to be administered to the perfusion device.

Table 1: Components of the perfusion fluid.[16]

Components	Quantity
Packed red blood cell (Hematocrit 60%)	840 ml
Fresh frozen plasma	930 ml
Human albumin 200 g/L (Albuman, Sanquin)	100 ml
Modified parenteral nutrition (Clinimix N17G35E, Baxter International Inc.)	7.35 ml
Multivitamins for infusion (Cernevit, Baxter international Inc.)	7 µl
Concentrated trace elements for infusion (Nutritrace , B. Braun Melsungen AG)	7.35 ml
Metronidazol for i.v. administration (5 mg/ml) (Flagyl, Sanofi-Aventis)	40 ml
Cefazolin 1,000 mg flask 5 ml powder for i.v. administration (Servazolin,	2 ml
Fast-acting insulin (100 IU/ml) (Actrapid®, Novo Nordisk)	20 ml
Calcium glubionate, intravenous solution 10%, 137.5 mg/ml (Sandoz)	40 ml
Sterile H_2O	51.3 ml
NaCl 0.9% solution	160 ml
Sodium bicarbonate 8.4% solution	31 ml
Heparin 5,000 IE/ml for i.v. administration	4 ml
Total	2,233 ml

2. Priming of the Perfusion Device

a. Add the components of the perfusion fluid, including the human packed red blood cells, fresh frozen plasma, human albumin and the solution prepared in step 1.1 to the machine via the connector on top of the oxygenators and remove all the air bubbles from the tubing.
b. Switch on the venous pump and follow the manufacturer's instructions on the screen. Then turn on the arterial pump and follow the manufacturer's instructions on the screen.
c. Null the pressure meters against atmospheric pressure by following the instructions on the screen. This ensures that the pressure measured during the perfusion is the real pressure at the level of the portal vein and the hepatic artery.
d. Start the oxygenation using carbogen (95% O_2 + 5% CO_2) at a flow rate of 4 L/min. The air flow will be divided among the two oxygenators (2 L/min per oxygenator) and this should result in a $pO_2$of around 60 kPa (or 450 mmHg) in the perfusion fluid. For longer perfusions, it is advisable to use separate sources of oxygen and carbon dioxide. This allows for small adjustments in the O_2/CO_2ratio, which can be used to adjust the pH and pCO_2 of the perfusion fluid.
e. Take a perfusion sample for blood gas measurement 15-20 min after the device has been primed and monitor the pH and electrolytes accordingly. NOTE: Be sure to discard about 3 ml of perfusion fluid before taking the samples, as this fluid is in the peripheral tubing and does not represent the perfusion fluid in the system. Add an 8.4% sodium bicarbonate solution for buffering capacity, aiming for a physiological pH (7.35-7.45). For example, add 25-35 ml of an 8.4% sodium bicarbonate solution and check the pH and bicarbonate levels in the perfusion fluid by taking samples for blood gas measurement at regular intervals.

3. Procurement and Preparation of Donor Livers

Note: Procure the organ using the standard technique of *in situ* cooling and flush out with cold preservation fluid (0-4 °C)[19]. To facilitate cannulation of the artery, leave a segment of the supratruncal aorta attached to the hepatic artery (**Figure 2A**).

a. Flush out the bile ducts with the preservation fluid (*i.e.,* University of Wisconsin solution). Ligate the cystic duct with a surgical suture.
b. Pack and store the organ in a standard sterile donor organ bag and box with crushed ice for subsequent transportation to the MP center.
c. Start the back table procedure immediately upon arrival of the donor liver in the operating room.
    1. Take a sample of at least 10 ml of the preservation fluid for microbiological testing.

2. Remove the diaphragmatic attachments to the bare area of the liver as well as any remaining cardiac muscle from the upper cuff of the vena cava with surgical scissors.
3. Dissect the artery and portal vein using dissecting scissors and ligate side branches using surgical sutures or hemoclips.
4. Close the distal end of the supratruncal aorta segment using a non-absorbable monofilament suture (*e.g.,* 3-0 Prolene). Insert the arterial cannula into the proximal end of the supratruncal aorta and secure with sutures (**Figure 2A**). Use the cannula provided in the disposable package as supplied by the manufacturer of the perfusion device.
5. Insert the venous cannula in the portal vein and secure with sutures. Use the cannula provided in the disposable package. The hepatic vein remains uncannulated.
6. Flush out the bile duct with the preservation solution. Insert a silicon catheter into the bile duct and secure with sutures. NOTE: Do not insert the catheter too deeply into the bile duct as this may cause injury to the biliary epithelium.
7. Flush out the liver with 0.9% NaCl solution via the portal vein cannula as follows:
    a. If the graft has been preserved in University of Wisconsin solution as the preservation solution, flush out the liver with 2,000 ml of cold (0-4 °C) 0.9% NaCl solution followed by 500 ml of warm (37 °C) 0.9% NaCl solution.
    b. If the graft has been preserved in Histidine-Tryptophan-Ketoglutarate solution as the preservation solution, flush out the liver with 1,000 ml of cold (0-4 °C) 0.9% NaCl solution followed by 500 ml of warm (37 °C) 0.9% NaCl solution. The purpose of the warm flush is to prevent a significant drop in the temperature of the perfusion fluid.
    c. Perform the warm flush immediately before connecting the liver to the perfusion device. NOTE: Always keep the duration between warm flush and start of NMP less than 1-2 min.

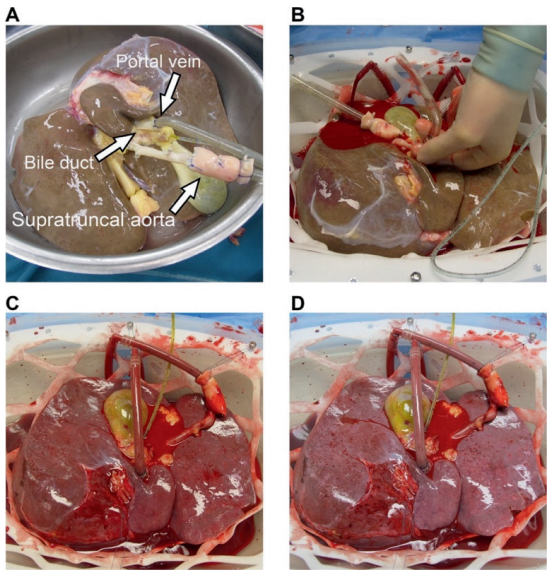

Figure 2: (**A**) Pictures of a human donor graft that has been prepared on the back table and (**B**-**D**) was subsequently perfused normothermically. (**A**) The arterial cannula is inserted into the surpratruncal aorta and the venous cannula is inserted into the portal vein. The bile duct is cannulated with a silicon biliary catheter. (**B**) The liver is positioned in the organ chamber with its anterior surface facing downwards and cannulas are connected to the tubings of the perfusion device. (**C**) 30 min after the start of normothermic machine perfusion. (**D**) 6 h after the start of normothermic machine perfusion. During operation the organ chamber is covered by a transparent cover to maintain a sterile moist environment for the liver (not shown in these pictures).

4. Normothermic Machine Perfusion

a. Position the liver in the organ chamber with the anterior surface facing downward. Immediately connect the liver to the primed perfusion device by connecting the portal vein cannula to the portal inflow tube of the perfusion device and the arterial cannula to the arterial inflow tube of the device.
b. Start perfusion on both portal and arterial side by following the manufacturer's instructions on the screen. Set the mean arterial pressure at 70 mmHg and the mean portal venous pressure at 11 mmHg.
c. Take perfusion fluid samples every 30 min for immediate analysis of blood gas parameters (pO_2, pCO_2, sO_2, HCO_2^- and pH) and biochemical parameters (glucose, calcium, lactate, potassium and sodium) using a conventional blood gas analyzer. Be sure to discard about 3 ml of perfusion fluid before taking the samples, as this fluid is in the peripheral tubing and does not represent the perfusion fluid in the system.
    a. To take these samples aspirate the perfusion fluid using a 1 ml syringe from the sampling connectors that are part of the disposable tubing set of the perfusion device. For each sample use a new syringe and

immediately remove any air bubbles from the syringe upon aspiration of perfusion fluid. Then insert the syringe in the blood gas analyzer and follow the manufacturer's instructions provided in the manual of the analyzer.

d. Collect plasma from the perfusion fluid, freeze and store at -80 °C for determination of alkaline phosphatase (AlkP), gamma-glutamyl transferase (gamma-GT), alanine aminotransferase (ALT), urea and total bilirubin. Collect plasma after 5 min of centrifugation of the perfusion fluid at 1,500 x g and 4 °C.

REPRESENTATIVE RESULTS

12 human livers that were declined for transplantation due to various reasons were used after obtaining informed consent for research from donor families. Donor characteristics are described in **Table 2**. The human donor livers were perfused normothermically for 6 h by using the protocol described in this paper. The quality of the liver grafts were evaluated by monitoring the macroscopic homogeneity of liver perfusion (**Figure 2A**-**D**). The hemodynamics of the livers were assessed by monitoring the changes in the arterial and portal flows. An initial increase in hepatic artery and portal vein flows and subsequent stabilization of the flows were observed, resulting in a mean arterial flow of 256 ± 16 ml/min (mean ± SEM) and a mean portal vein flow of 748 ± 34 ml/min (mean ± SEM) at 6 h, indicating stable hemodynamics of livers during perfusion (**Figure 3A**). Blood gas analysis of the perfusate samples collected from arterial perfusion fluid was used to monitor the status of oxygenation in the perfusion fluid. Oxygenation with carbogen (95% O_2 and 5% CO_2) at a flow of 4 L/min resulted in a continuous O_2 saturation of 100%. **Figure 3B** displays the oxygenation of the perfusion fluid and subsequent extraction of carbon dioxide in our experience.

Table 2: Donor characteristics.

Donor characteristics (N = 12)	**Number (%) or Median (IQR)**
Age (years)	61 (50-64)
Gender (male)	8 (67%)
Type of donor	
DCD, Maastricht type III	10 (83%)
DBD	2 (17%)
Body mass index (BMI)	27 (25-35)
Reason for rejection	
DCD+ age >60 years	5 (41%)
DCD+ high BMI	3 (25%)
DCD+ various reasons*	2 (17%)
Severe steatosis	2 (17%)
Preservation solution	
UW solution	6 (50%)
HTK solution	6 (50%)
Donor warm ischemia time in DCD (min)	14 (17 - 20)
Cold ischemia time (min)	389 (458-585)
Donor risk index (DRI)	2.35 (2.01-2.54)

* donor history of intravenous drug abuse for one graft and prolonged donor sO_2 <30% after withdrawal of life support for another graft. *Abbreviations: DCD, donation after circulatory death; DBD, donation after brain death; UW, University of Wisconsin; HTK, Histidine-tryptophan-ketoglutarate*

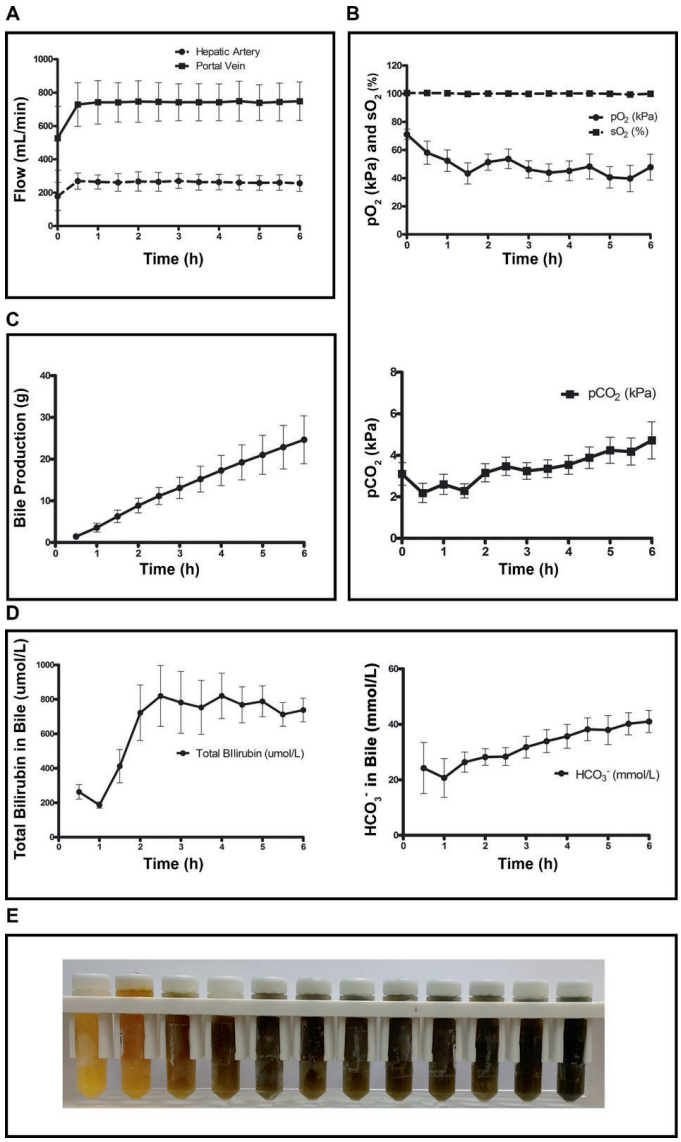

Figure 3: Graphical presentation of perfusion parameters and biochemical analyses of both the perfusion fluid and bile during 6 h of normothermic machine perfusion of 12 human livers. (**A**) Changes in arterial and portal flow. (**B**) Evolution of oxygenation characteristics and pCO_2 during 6 h of normothermic perfusion. (**C**) Cumulative bile production during perfusion. (**D**) Increasing concentrations of bilirubin and bicarbonate in bile samples taken during machine perfusion. (**E**) Microcentrifuge tubes containing bile from a representative graft, demonstrating a gradual darkening shade of the bile color over time. Data are expressed as mean ± SEM.

Bile production was used as an indicator of liver function. Metabolically functioning livers produced bile during NMP, resulting in a mean total bile production of 24.6 ± 6 g after 6 h of NMP (**Figure 3C**). An increase in the concentration of total bilirubin and bicarbonate in the bile represented an improvement in the quality of the bile produced during NMP (**Figure 3D, E**). Liver tissue ATP content as an indicator of mitochondrial function increased during NMP, resulting in mean ATP of 30 ± 5 µmol/g protein (mean ± SEM) after 6 h of NMP (**Figure 4**). Biochemical analysis of hepatic injury markers in the perfusion fluid, such as ALT, AlkP, gamma-GT and potassium, was used to assess the amount of graft injury. Stable concentrations of hepatic injury markers reflected minimal injury of the grafts during perfusion (**Figure 5A**). Lactate and glucose levels in the perfusion fluid as well as oxygen consumption have been described previously[17]. Furthermore, histological examination of H&E stained biopsies collected from liver tissue and the distal end of the extrahepatic bile duct, as illustrated in **Figure 5B, C** did not reveal any additional injury to the grafts during normothermic machine perfusion.

Microbiological testing of the perfusion fluid did not reveal any bacterial contamination during NMP. In one case a positive culture for *S. epidermidis* was obtained from the sample collected immediately after cold preservation. However, culture of the perfusion fluid after 6 h of NMP was negative for any bacteria, showing the efficacy of the antibiotics used in the perfusion fluid.

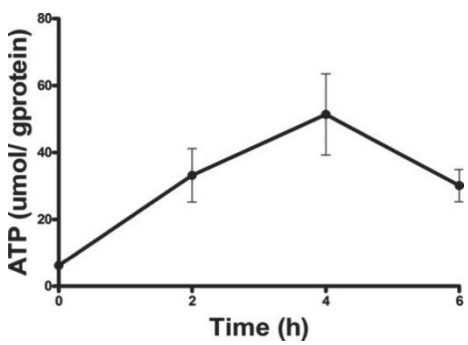

Figure 4: Changes in the level of liver tissue ATP content during NMP. Increased liver tissue ATP content during NMP showed improvement of mitochondrial function. Data are represented as mean ± SEM.

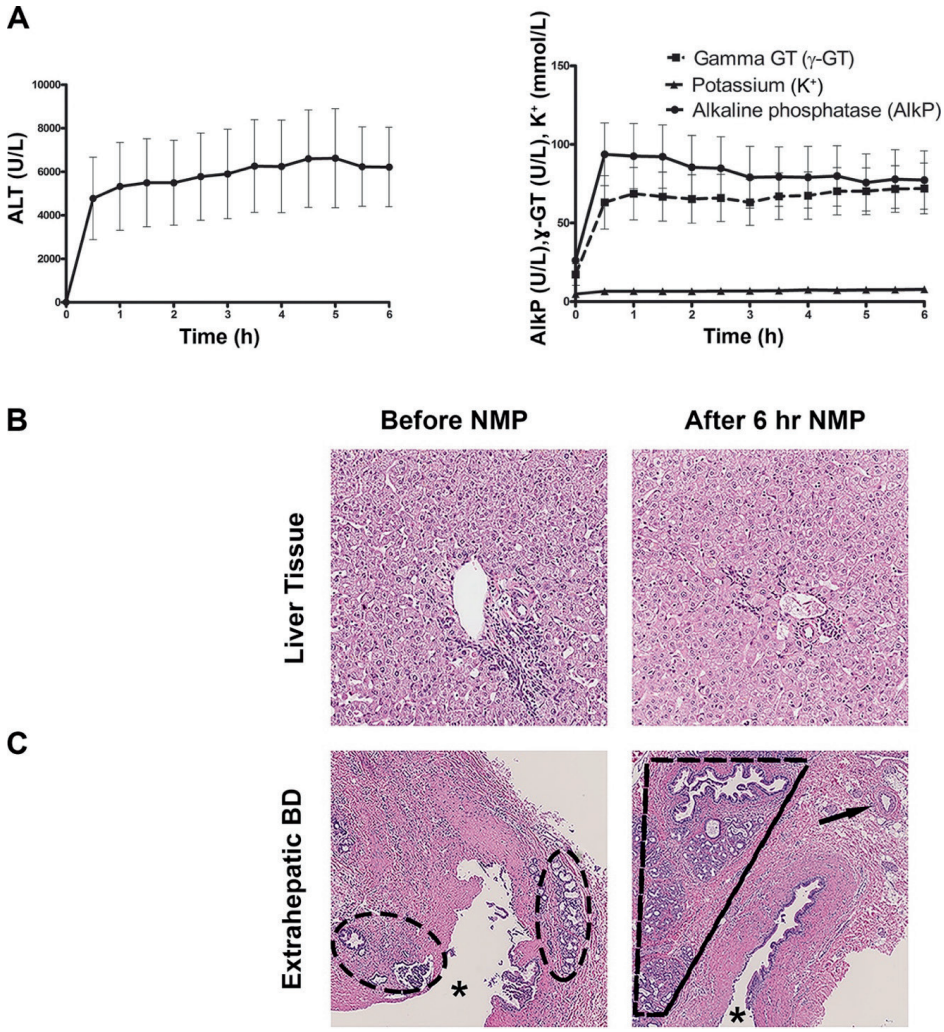

Figure 5: (**A**) Markers of hepatobiliary injury and (**B**) staining of liver parenchyma and (**C**) the extrahepatic bile duct taken from a representative graft before (0 h) and after (6 h) machine perfusion. (**A**) Stable concentrations of injury markers in the perfusion fluid indicated minimal injury of grafts during machine perfusion. (**B**) Well-preserved microscopic architecture of a representative liver graft. (**C**) Histology of the extrahepatic bile duct (lumen marked by an asterisk) of a representative graft. Moderate biliary epithelial injury indicated by partial loss of the luminal epithelial layer was observed at baseline and this did not worsen during 6 h of MP. A similar degree of biliary injury has been described in a series of human livers before transplantation.[20] Peribiliary vasculature (arrow) and peribiliary glands (area within dashed lines) displayed no worsening of injury after normothermic machine perfusion.

DISCUSSION

This video provides a step by step protocol for normothermic machine perfusion of human donor livers using a device that enables pressure controlled dual perfusion through the hepatic artery and portal vein. While following this protocol, technical failures of the perfusion machine did not occur and all grafts were well perfused and well oxygenated. The *ex situ* perfused livers had stable hemodynamics and were metabolically active, as defined by the production of bile.[16,17]

This is a well-established protocol for machine perfusion of human donor livers. This technique has several potential advantages over the conventional method of SCS[21]. Machine perfusion provides the opportunity to preserve donor liver grafts at different temperatures depending on the intended endpoint of organ preservation. Hypothermic oxygenated machine perfusion provides better perfusion and wash-out of the microvasculture and may help to restore intracellular energy contents by stimulating adenosine triphosphate (ATP) regeneration. However, full assessment of graft viability requires perfusion at a more physiological temperature (subnormothermic or normothermic). With increasing perfusion temperatures, the liver will become metabolically more active and start to produce bile. A recent study has suggested that bile production as an indicator of liver function might be an asset during *ex situ* NMP to evaluate graft viability prior to transplantation. This study showed that bile production correlated with the liver tissue ATP level and histological and biochemical markers of liver injury.[17] These findings remain to be confirmed by clinical trials. Although bile production is a suitable potential marker of liver parenchyma viability, markers of bile duct viability that can be assessed during *ex situ* NMP are still lacking. Therefore, it is currently still not possible to predict whether a liver assessed during NMP will develop NAS after transplantation or not. However, using this protocol, *ex situ* NMP did not reveal any worsening of bile duct injury during 6 hours of NMP. Moreover, this technique has the potential to allow for preconditioning of the graft before transplantation, resulting in reduced post-transplant injuries or recurrence of underlying diseases.[22]

The optimal fluid for *ex situ* oxygenated machine perfusion of donor livers is dependent on the temperature used. The solubility of oxygen in water is temperature-dependent and the amount of oxygen that can be dissolved in a watery fluid decreases with increasing temperature.[23] When using low temperatures for MP, the amount of oxygen dissolved in the perfusion fluid can be sufficient. However, at 37 °C an oxygen carrier should be added to the perfusion fluid to provide enough oxygen to the graft. For hypothermic MP, a preservation solution such as Belzer Machine Perfusion Solution can be sufficient.[11] For subnormothermic or normothermic MP, more complex perfusion fluids that also contain nutrients and an oxygen carrier have been used in different studies.[15,16] In our studies on normothermic MP, we have used ABO- and Rhesus matched packed red blood cells from the local blood bank as an

oxygen carrier.[16] It remains to be established whether similar results can be obtained with artificial hemoglobin-based oxygen carriers such as Hemopure or Hemarina.

The most critical technical aspects for successful perfusion of human livers are: to correctly secure the cannulas in the portal vein and supratruncal aorta segment, to ligate all small side braches to avoid any leakage of perfusion fluid which could disturb the pressure and flow regulations of the machine, to maintain a physiological environment for the liver especially by adjusting the pH and electrolyte concentrations of the perfusion fluid, and to maintain sterility of the perfusion environment.

Due to technical constraints, the perfusion device used in the described protocol cannot lower the temperature of the perfusion fluid below 10 °C. Although this can be considered a limitation, it does not provide a real problem concerning ischemia. The reason is that more than sufficient amounts of oxygen can be supplied to the perfusion fluid by the two membrane oxygenators regardless of the temperature. An advantage is that the temperature can be easily adjusted during the perfusion period, which allows gradual rewarming of the donor liver. A recent study in porcine livers has shown important advantages of gradual rewarming prior to normothermic reperfusion using the same device as described here.[18]

The ability to perfuse donor livers at different temperatures and the opportunity of adding extra agents to the perfusion fluid during organ perfusion offer the potential to assess and improve organ quality prior to transplantation. Therefore, this method can considerably increase the number of available organs for transplantation.

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